Structure-function relations of strigolactone analogs: Activity as plant hormones and plant interactions

Molecular Plant • Volume 6 • Number 1 • Pages 141–152 • January 2013 RESEARCH ARTICLE

Structure–Function Relations of Strigolactone Analogs: Activity as Plant Hormones and Plant Interactions

Maja Cohen[a,b], Cristina Prandi[c], Ernesto G. Occhiato[d], Silvia Tabasso[c], Smadar Wininger[a], Nathalie Resnick[a], Yosef Steinberger[b], Hinanit Koltai[a] and **Yoram Kapulnik[a1]**

a Institute of Plant Sciences, ARO, Volcani Center, Bet Dagan, Israel
b The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel
c Dipartimento di Chimica, Università di Torino, via P. Giuria 7, 10125 Torino, Italy
d Dipartimento di Chimica 'Ugo Schiff', Università degli Studi di Firenze, via della Lastruccia 13, Sesto Fiorentino, Firenze, Italy

ABSTRACT Strigolactones (SLs) have several functions as signaling molecules in their interactions with symbiotic arbuscular mycorrhizal (AM) fungi and the parasitic weeds *Orobanche* and *Striga.* SLs are also a new class of plant hormone regulating plant development. In all three organisms, a specific and sensitive receptor-mediated perception system is suggested. By comparing the activity of synthetic SL analogs on *Arabidopsis* root-hair elongation, *Orobanche aegyptiaca* seed germination, and hyphal branching of the AM fungus *Glomus intraradices*, we found that each of the tested organisms differs in its response to the various examined synthetic SL analogs. Structure–function relations of the SL analogs suggest substitutions on the A-ring as the cause of this variation. Moreover, the description of competitive antagonistic analogs suggests that the A-ring of SL can affect not only affinity to the receptor, but also the molecule's ability to activate it. The results support the conclusion that *Arabidopsis*, *Orobanche*, and AM fungi possess variations in receptor sensitivity to SL analogs, probably due to variation in SL receptors among the different species.

Key words: strigolactone; *Arabidopsis*; *Orobanche*; AM fungus *Glomus intraradices*; root hair; seed germination; hyphal branching.

INTRODUCTION

Strigolactones (SLs) are a group of sequisterpene lactones produced in plants (Matusova et al., 2005). SLs were initially identified as germination stimulants of the parasitic plants *Striga* (witchweed) and *Orobanche* (broomrape) (Cook et al., 1966, 1972; Hauck et al., 1992; Müller et al., 1992; Butler, 1995), and later were shown to be involved in communication between plants and the symbiotic arbuscular mycorrhizal (AM) fungi (Akiyama et al., 2005; Besserer et al., 2006; Akiyama, 2007). Both AM fungi and seeds of parasitic plants respond to very low concentrations (10^{-12} M) of SL (Besserer et al., 2008; Kim et al., 2010), suggesting a highly sensitive perception system mediated by a receptor in both biological systems.

Recently, SLs were shown to belong to a new class of hormones (Gomez-Roldan et al., 2008; Umehara et al., 2008). They are mainly produced in the roots, suggested to be transported upward from the roots through the xylem (Kohlen et al., 2011), and inhibit shoot lateral bud outgrowth (reviewed by Dun et al., 2009). Aside from their activity in the regulation of aboveground architecture, SLs affect root growth and root system architecture by repressing lateral root formation and promoting root-hair (RH) elongation (Koltai et al., 2010; Kapulnik et al., 2011; Ruyter-Spira et al., 2011; reviewed by Koltai (2011)). Mutants impaired in their SL response have been found in several plant species, including *Arabidopsis thaliana* (Stirnberg et al., 2002), pea (*Pisum sativum*) (Beveridge et al., 1996), and rice (*Oriza sativa*) (Ishikawa et al., 2005). MAX2/RMS4/D3, which encodes an F-box protein (Stirnberg et al., 2007), is suggested to be one of the plausible players in the SL signaling pathway (Umehara et al., 2008).

To date, more than 15 naturally occurring SLs have been isolated from root exudates of several plant species (reviewed by Xie et al. (2010)). All of the identified active SL molecules

[1] To whom correspondence should be addressed. E-mail kapulnik@agri.gov.il, tel. 03–968–3461, fax 03–968–1492.

doi:10.1093/mp/sss134, Advance Access publication 8 December 2012

Received 20 August 2012; accepted 11 November 2012

present a tricyclic ring system (ABC-ring) linked to a butenolide ring (D-ring) via an enol ether bond. Three molecular mechanisms of SL binding to its receptor in seeds of parasitic weeds have been proposed: the first consists of a Michael addition to the enol ether linking the C- and D-rings (Mangnus and Zwanenburg, 1992; Zwanenburg et al., 2009). According to more recent hypotheses, the D-ring itself plays a crucial role, undergoing simple lactone hydrolysis (Scaffidi et al., 2012) or direct nucleophilic attack of the butenolide double bond (Zwanenburg and Mwakaboko, 2011; Scaffidi et al., 2012).

Our understanding of the molecular mechanisms leading to SL activity is, however, still very incomplete. One way to provide new insight into this field is by studying additional natural or synthetic SL analogs (Thuring et al., 1997; Reizelman et al., 2003) and their connection to added SL biological functions. Recent studies have described the synthesis of new fluorescent SL analogs (Bhattacharya et al., 2009; Prandi et al., 2011). These newly synthesized molecules (Prandi et al., 2011) are nitrogen derivatives and present different models of substitution on the A-ring; moreover, the C-ring is a ketone (i.e. the EGO and ST series) instead of a lactone as in the natural molecules or in the most common synthetic analogs. These new analogs present different levels of activity on AM fungus hyphal branching and on weed seed germination (Prandi et al., 2011).

For better insight into the structural requirements of SL relative to its different functions, we selected, among the high number of synthetic analogs available, molecules that share similar BCD structures but differ in the composition of their A-ring. This allowed us to determine the effect of the identity of the A-ring on SL activity. In this way, we could identify their variation in activity, isolate its potential cause, and decipher some of the structure–function relations of these SL analogs.

The activity and competitive behavior of the selected, newly synthesized SL analogs (Prandi et al., 2011) were analyzed with respect to *A. thaliana* RH elongation and germination of the parasitic weed *Orobanche aegyptiaca*; these results were further compared with the analogs' effect on hyphal branching of the AM fungus *Glomus intraradices*. The results showed variations in receptor sensitivity to SL analogs in *Arabidopsis*, *Orobanche*, and AM fungi. These variations might result from differences in the SL receptors, suggesting the existence of either different receptors leading to different perception systems or differences in receptor-binding sites among the different species. The different patterns of substitution and the nature of the substituents, particularly on the A-ring, play a crucial role in determining SL affinity and their intrinsic activity in each of the examined system.

RESULTS

Strigolactone Analogs: Synthesis and Structural Features

For the present study, the following molecules were chosen: GR24 as a reference; GR5 and GR7 because of the simple structure lacking the AB- and A-ring, respectively; from the indolyl series, EGO10 because it is the simplest analog in the series and it exhibits high activity in both seed germination and hyphal branching experiments (Prandi et al., 2011); EGO23 because of its voluminous side group attached to the A-ring; and ST357 and ST362 because they present a thiophene and diethylenedioxythiophene on the A-ring.

Strigolactone analogs EGO23, EGO10, ST357, and ST362 were synthesized according to the recently reported synthetic sequences (referred to as EGO9b, EGO5, ST23c, and ST28, respectively, in Prandi et al. (2011); Figure 1). All of them are indolyl derivatives, with the C-ring in the ST series being constructed by Nazarov electrocyclization. The stereochemistry at the enol ether double bond has been tentatively assigned as *E* (Noesy experiments) in accordance with data from the literature (Zwanenburg et al., 2009).

The main difference between the two sets, ST and EGO, lies in the presence of a methyl group on the C-ring of the ST derivatives. This implies that, in the ST series, in addition to C2', another stereocenter is present and the molecule is thus obtained as a mixture of four stereoisomers. The EGO series analogs, possessing only C2' as the stereocenter, are obtained instead as racemic mixtures. With respect to the substitution on the A-ring, EGO10 is the simplest molecule used in this set. ST357 possesses a thiophene at position 7 on the A-ring, ST362 a 1,2-ethylenedioxythiophene, and EGO23 a *p*-dimethylaminophenyl group at the same position on the A-ring. The substituents on the A-ring thus differ with respect to both bulkiness and hydrophobicity—the dimethylamino group on EGO23 is more polar than thiophene or 1,2-ethylenedioxythiophene (Figure 2).

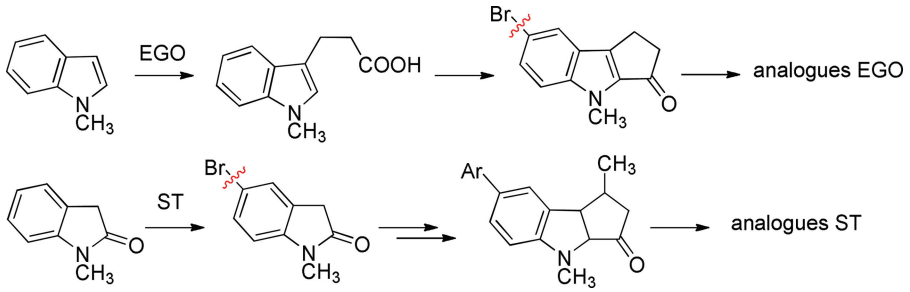

Figure 1. Synthesis of ST and EGO Series SL Analogs.

Synthesis of the SL analogs GR5, GR7, and GR24 was as previously reported by Johnson et al. (1976, 1981).

Biological Activity of SL Synthetic Analogs

Activity of the SL analogs was examined by three biological assays: germination of the parasitic plant *O. aegyptiaca* (Cook et al., 1972), hyphal branching of the AM fungus *G. intraradices* (LPA 8; Akiyama et al., 2005), and *A. thaliana* (Col-0) RH elongation (Kapulnik et al., 2011).

Orobanche *Seed Germination*

Sensitivity of *O. aegyptiaca* seeds to the different SL analog molecules was determined at six different analog concentrations. The analogs' dose–response curves showed the sigmoidal shape typical of a receptor-binding curve, where the maximum response reflects the intrinsic activity of the compound and the EC_{50} value reflects its affinity (Weyers et al., 1987). The sigmoidal pattern of the GR24 curves shows the positive effect of increasing concentrations of SL analogs on their affinity to the receptor (cooperative binding). In contrast, ST357 (Figure 3A) displayed a flatter and less sigmoidal dose–response curve than GR24. This is representative of less cooperative binding, where the affinity for the ligand (ST357) is not enhanced by increasing its concentration. Thus, these results suggest that the affinity to the receptor is different between ST357 and GR24 (Johnson et al., 1981). However, both molecules (Figure 3A) induced a maximum 80% *Orobanche* seed germination, and they therefore have the same intrinsic activity. At any given concentration, seeds exposed to ST362 had the lowest germination rate; the maximum response induced by this molecule reached only half of the maximum germination rate obtained with the other analogs (Figure 3A). Due to this low intrinsic activity, ST362 can be considered a partial agonist. In contrast, EGO10 and GR24 presented similar curve patterns and maximum response values (around 80%). However, EGO10 showed a higher EC_{50} value (1.4×10^{-7} M) than GR24 (1.1×10^{-8} M), resulting in a rightward shift of the curve with respect to GR24 (Figure 3B). EGO23 did not reach the plateau phase in this concentration range, preventing a determination of the maximum response and EC_{50} value. The different SL analogs therefore exhibited various levels of intrinsic activity and affinity to the putative receptor of *O. aegyptiaca*, as a result of their distinct chemical structures.

Mycorrhizal Hyphal Branching

The different analogs were tested on hyphal branching of the AM fungus *G. intraradices*. The analogs were applied at 10^{-7} M, a concentration known to induce a high branching response with the analog GR24 (Akiyama et al., 2005). Differences were observed between the molecules in their effects on AM hyphal branching. ST362 was found to strongly promote hyphal branching and was almost as active as the positive control GR24; a pronounced effect of the synthetic SL ST362 was found on second-order hyphal branching, in contrast to the SL analog ST357, which induced the weakest effect on the branching of second-order hyphae of *G. intraradices* (Figure 4). Thus, the AM fungal response to the SL analogs is structure-specific and differs from that of *O. aegyptiaca*.

RH Length

Kapulnik et al. (2011) reported that GR24 application on WT *Arabidopsis* seedlings leads to an increase in RH length. Seedlings of *A. thaliana* WT were therefore exposed to the SL analogs at concentrations varying from 10^{-11} M to 10^{-5} M. RH

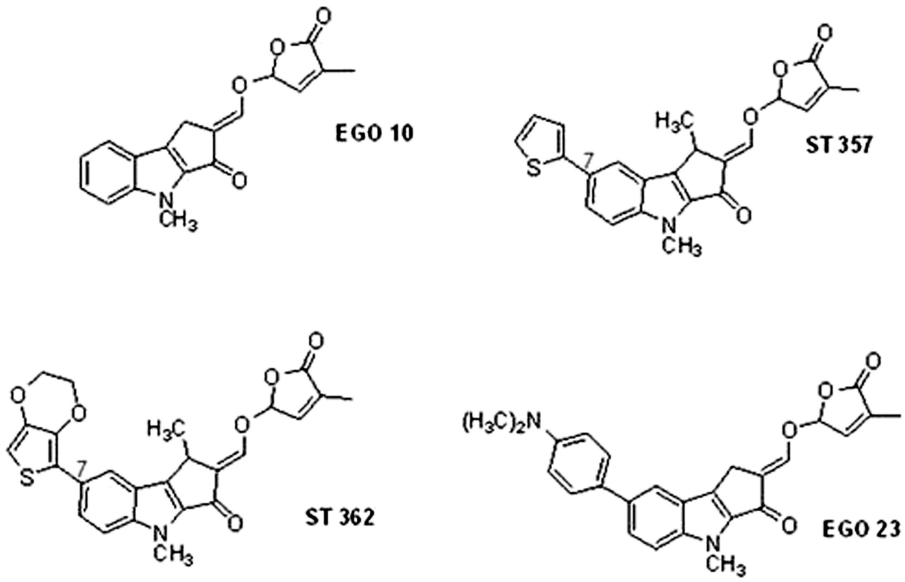

Figure 2. Structure of the Synthetic SL Analog Molecules.

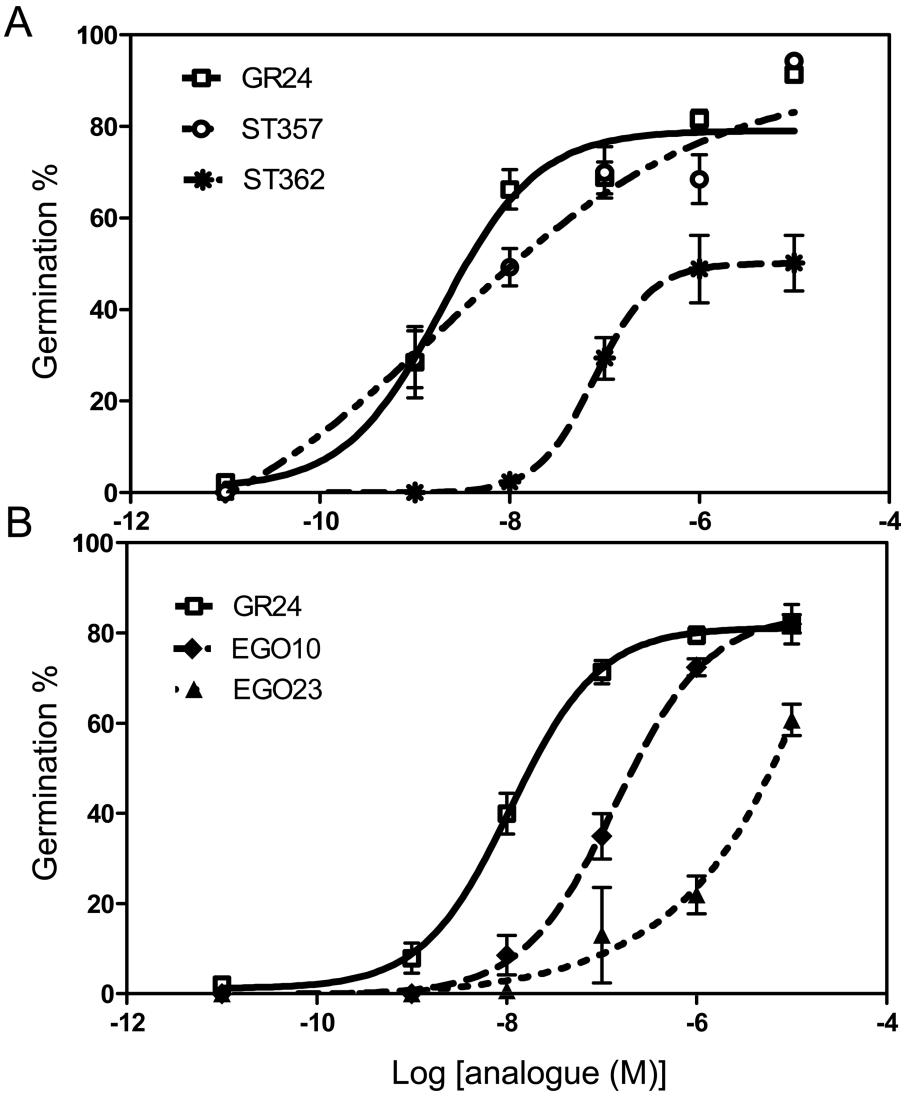

Figure 3. Germination Rate of *Orobanche aegyptiaca* Seeds Exposed to Increasing Concentrations of Synthetic Analogs Spaced on a Logarithmic Scale.

(A) ST357 and ST362.

(B) EGO10 and EGO23.

For each treatment, 12 replicates with 30–40 seeds per replicate were tested with 40 µl of test solutions for 1 week. The experiment was repeated three times. Mean (n = 350 seeds) ± SE is shown. Acetone 0.1% was used as negative control and leads to germination rates of 0.7% ± 0.1% (not shown in the graph). The logistic dose–response curves were obtained by nonlinear regression using GRAPHPAD-PRISM 5 software with variable Hill coefficient.

lengths were evaluated 48 h post exposure to the different treatments and controls. The *A. thaliana* RH-elongation response to the analog molecules was concentration-dependent (Figure 5). The SL analog EGO10 showed a curve pattern similar to that obtained following GR24 exposure at the same concentrations: EGO10 and GR24 induced elongation of RHs to a maximum 2.5-fold that of the untreated control (Figure 5A). The SL analog EGO23 induced RH elongation more efficiently than any of the other analogs, but did not reach saturation under the examined concentrations. In contrast, *Arabidopsis* roots were completely insensitive to the ST series of analog molecules with respect to RH elongation, even at relatively high concentrations (Figure 5B).

The SL analogs were tested on the *Arabidopsis* MAX2 mutant which is impaired in SL signaling and, similarly to GR24 (Kapulnik et al., 2011), they did not lead to a RH-elongation response (Supplemental Figure 1). These results indicate that the SL analogs act through the same MAX2-mediated signaling pathway as GR24.

To address the role played by the AB part of SL and its substitution in RH elongation, we determined the RH response in seedlings of *A. thaliana* to the SL analogs GR24, GR5, and GR7. GR5 and GR7 are widely used to define the minimal structural requirements for SL. Indeed, it has been previously determined (Akiyama et al., 2010; not shown) that the CD part of the SL molecule is not sufficient to induce hyphal

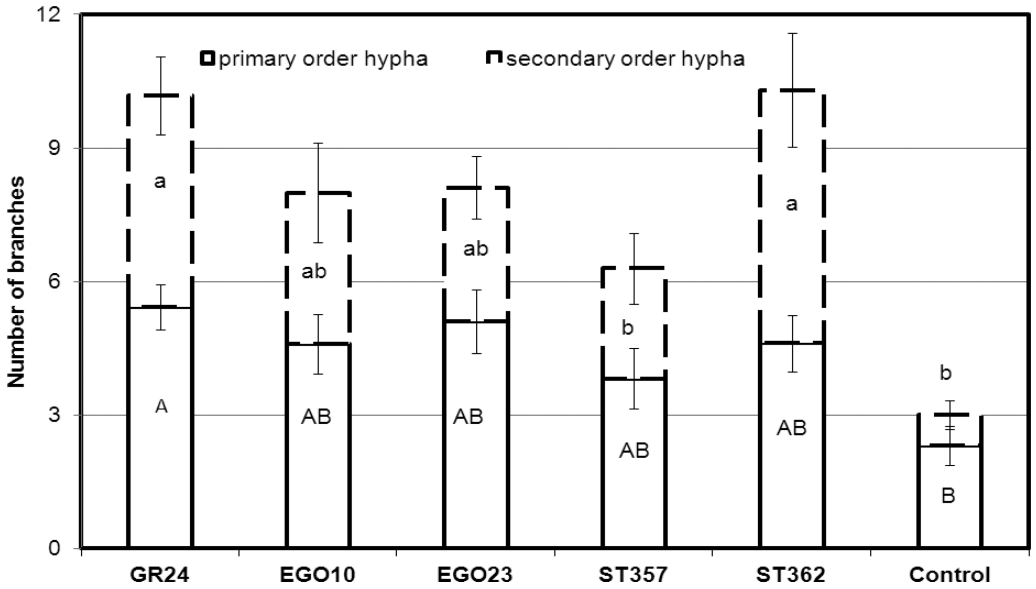

Figure 4. Hyphal Branching of AM Fungus *Glomus intraradices* Spores Exposed to 3×10^{-7} M of the Different Analogs.
GR24 served as a positive control and 0.1% acetone as a negative control. The activity of each analog was determined by its ability to induce branching of the first order (branches growing from the primary hypha) and of the second order (growing from the first-order branches). The experiment was repeated three times in three replicates with five germinating spores each. Mean (n = 15 spores) ± SE is shown. Different capital letters indicate significantly different means in the first-order branching and different lower-case letters indicate significantly different means in the second-order branching (Tukey test; $P < 0.05$).

branching. However, GR5 and GR7 are able to induce *Striga* and *Orobanche* seed germination (Johnson et al., 1976) and to repress shoot-bud outgrowth (Boyer et al., 2012). Here, we examined the effects of GR5 and GR7 on RH elongation. All of the seedlings treated with GR5 and GR7 analogs at concentrations ranging from 3×10^{-8} M to 10^{-6} M showed elongated RHs compared to the negative control (Figure 6). Moreover, the *Arabidopsis* responses to GR5, GR7, and GR24 at each concentration were similar. The high effectiveness of the truncated SL analogs GR5 and GR7 support the conclusion that the C- and D-rings are enough to induce SL activity in roots of *Arabidopsis*; these results further suggest that the active site of the molecule resides in its CD part.

Competitive Behavior of SL Analogs in *Orobanche* and *Arabidopsis*

Orobanche

As already noted, ST362 acts as a partial agonist in seeds of *O. aegyptiaca*; however, it is not clear whether the observed variation in intrinsic activity of ST362 reflects a different receptor subtype or a varying degree of receptor–effecter coupling. To delve into the nature of the interactions between the analog ST362 and the SL receptor of *O. aegyptiaca* seeds, we exposed seeds of *Orobanche* to increasing concentrations of GR24 together with a steady concentration of ST362 (Figure 7); ST362 was applied at 10^{-6} M, a concentration that normally induces 35% seed germination (almost half the maximum germination rate generated by GR24). As seen earlier, GR24 alone presented a sigmoidal, concentration-dependent response curve. However, in the presence of ST362, the graph showed a different pattern which can be analyzed in two parts: first, at low concentrations of GR24 (from 10^{-11} M to 10^{-8} M), ST362 had an additive effect, inducing a higher germination rate than GR24 alone (30% versus 1% and 40% versus 13% for 10^{-11} M and 10^{-9} M GR24, respectively). Second, once GR24 was present at concentrations (10^{-7} M and higher) capable of inducing germination more efficiently than 10^{-6} M ST362, the latter's additive effect progressively decreased. The germination rate normally obtained with GR24 was significantly reduced by the presence of ST362: at a concentration of 10^{-5} M, GR24 alone promoted 79% ± 1.6% germination, whereas, in the presence of the antagonist ST362, GR24 induced only 65% ± 4% germination. Hence, ST362 can be considered a partial agonist and a competitive antagonist of GR24 to the SL receptor of seeds of *O. aegyptiaca*.

Arabidopsis

To determine whether the inefficiency of the ST analogs in inducing RH elongation of *Arabidopsis* stems from either their low affinity or their inability to activate the process, we compared induction of RH elongation in the presence of the full agonist (GR24) with that in the presence of different concentrations of the ST analogs. GR24 was applied at a constant concentration, namely its EC_{50} concentration (3×10^{-7} M), and was mixed, before application to the plant, with ST362 or ST357 at increasing concentrations. Both of these analog molecules, when added to the growth medium, decreased the normal activity of GR24 in a concentration-dependent manner. From 10^{-6} M and up,

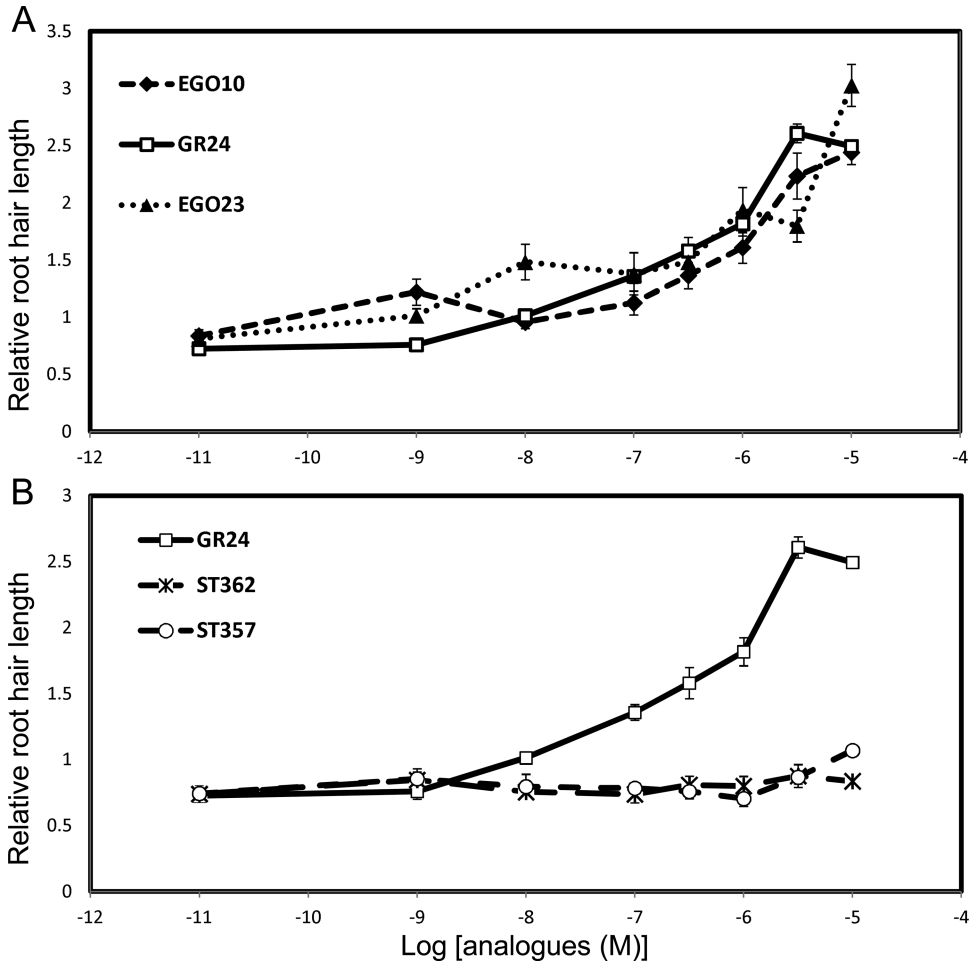

Figure 5. Root-Hair (RH) Elongation of *Arabidopsis thaliana* Col WT after 48 h of Exposure to Increasing Concentrations of Analogs.
(A) EGO10 and EGO23.
(B) ST362 and ST357. GR24 served as a positive control. The results are relative to the negative 0.1% acetone control: all RH length values were divided by the negative acetone control RH values, and are therefore relative values. RH lengths were measured on 15 seedling roots per treatment, 15–20 RHs per root. The experiment was repeated three times. Mean (n = 15 seedlings) ± SE is shown.

ST357 and ST362 interfered with GR24 activity and significantly reduced its effect (Figure 8A and B, respectively). Thus, ST362 and ST357 may be considered competitive antagonists of the putative SL receptor in *A. thaliana* that leads to the SL-induced RH-elongation response.

DISCUSSION

We focused on the biological activity of several SL analogs as effectors of *O. aegyptiaca* seed germination, *G. intraradices* hyphal branching, and *Arabidopsis* RH elongation. The competitive behavior of the analogs was examined in *Orobanche* and *Arabidopsis*. The results indicate differences between *Arabidopsis*, *Orobanche*, and AM fungi in their sensitivity to the SL analogs. These observations suggest the existence of either different receptors leading to difference perception systems or differences in receptor-binding sites among the different species. In addition, the structure–activity relations of the molecules and the critical effect of the A-ring substituent on SL affinity and intrinsic activity might be different in each system.

Previous studies have been conducted on SL structure–activity relationships in *Orobanche*, AM fungi, and shoot-bud outgrowths in plants (Zwanenburg et al., 2009; Akiyama et al., 2010; Boyer et al., 2012). The structural requirements of SL for *Orobanche* seed germination, hyphal branching of AM fungi, and shoot-branching inhibition in pea were found to be similar but not identical (Zwanenburg et al., 2009; Akiyama et al., 2010; Boyer et al., 2012). However, those studies did not directly compare all three biological systems (i.e. parasitic plant germination, AM fungus hyphal branching, and plant development) with respect to the existence of different perception schemes. Moreover, the current study extended the observations of the effects of different analogs on *Arabidopsis* RH elongation, which had only been determined for GR24 (Kapulnik et al., 2011).

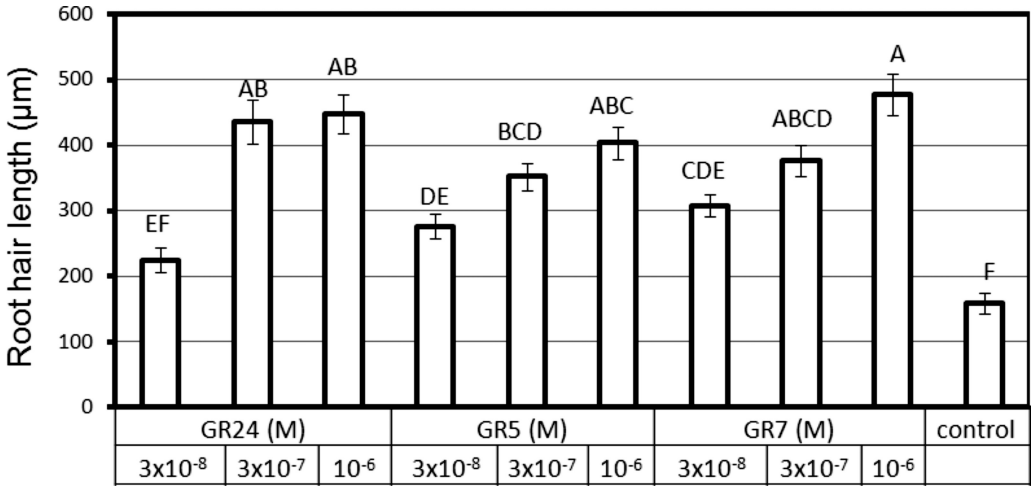

Figure 6. Root-Hair (RH) Elongation of *Arabidopsis thaliana* Col WT Seedlings after 48 h of Exposure to GR5 and GR7 at Concentrations from 3×10^{-8} M to 10^{-6} M.
GR24 was used as a positive control and 0.1% acetone as a negative control. RHs were measured on 15 seedling roots per treatment, 15–20 RHs per root. The experiment was repeated three times. Mean (n = 15 seedlings) ± SE is shown. Different letters indicate significantly different means (Tukey test; $P \leq 0.05$).

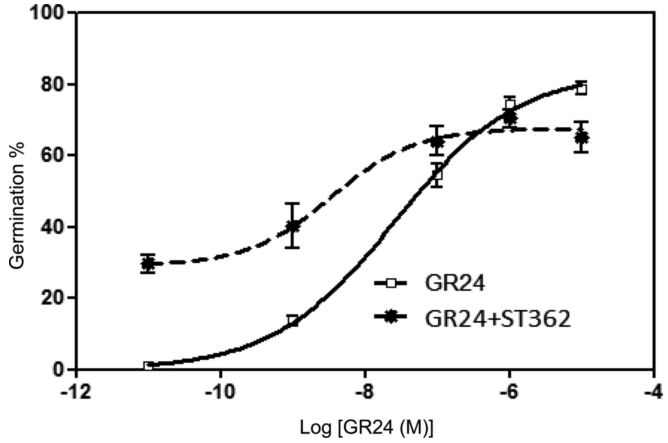

Figure 7. Germination Rate of *Orobanche aegyptiaca* Seeds Exposed to Increasing Concentrations of GR24 in the Presence of 10^{-6} M of the Analog ST362.
Twelve replicates with 30–40 seeds were tested per treatment with 40 µl of test solutions for 1 week. The experiment was repeated three times. Mean (n = 350 seeds) ± SE is shown. Acetone 0.1% was used as negative control and leads to germination rates of 0.7% ± 0.1% (not shown in the graph). The logistic dose–response curves were obtained by nonlinear regression using GRAPHPAD-PRISM 5 software with variable Hill coefficient.

Selection of the SL analogs reported here was based on their particular chemical structures and their specific activities in the different biological systems, as detailed above. The chemical structure of the analogs allowed discerning the effect of the A-ring's composition on SL activity. The analogs EGO23 and ST362 present voluminous and very specific side groups attached to the A-ring. In contrast, EGO10 and ST357 share the same BCD part with EGO23 and ST362, respectively, but present a reduced A-ring. Hence, comparing these analogs allows exploration of the effects of A-ring size and composition on their activity. Moreover, the tested organisms diverged in their response to the SL analogs. Based on this direct comparison of the effects of the SL analogs in the three biological systems, we could decipher some of these molecules' structure–activity relationships.

Different Levels of Sensitivity for Different Organisms

Direct comparison of the effects of different analogs on *Arabidopsis* RH elongation, *Orobanche* seed germination, and *G. intraradices* hyphal branching clearly revealed different behaviors in each of the tested organisms when exposed to the analogs. *Arabidopsis* roots were insensitive to thiophene-containing analogs (ST362 and ST357), whereas seeds of *O. aegyptiaca* were less sensitive to voluminous analog molecules (ST362 and EGO23). A thiophene-presenting voluminous molecule efficiently promoted hyphal branching of *G. intraradices* spores (ST362). These results suggest that

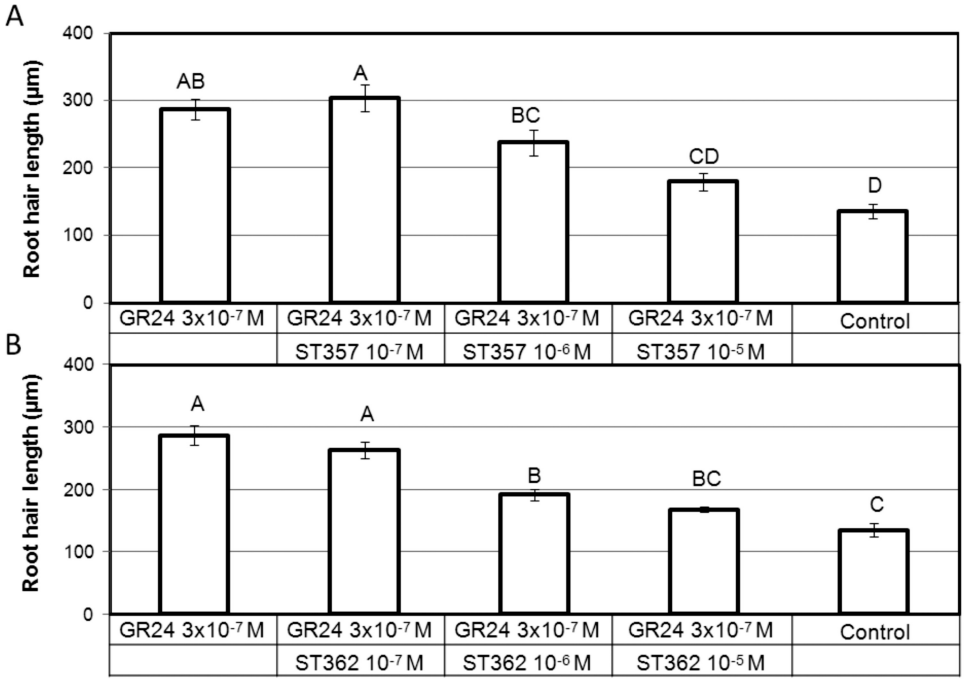

Figure 8. Root-Hair (RH) Elongation of *Arabidopsis thaliana* Col WT Seedlings after 48-h Exposure to a Constant Concentration of GR24 (3×10^{-7} M) Together with Increasing Concentrations of Synthetic Analogs which Are Unable to Promote RH Elongation.
(A) ST357.
(B) ST362. Positive and negative controls, 3×10^{-6} M GR24 and 0.1% acetone, respectively, are presented. RHs were measured on 15 seedling roots per treatment, 15–20 RHs per root. The experiment was repeated three times. Mean (n = 15 seedlings) ± SE is shown. Different letters indicate significantly different means (Tukey test; $P \leq 0.05$).

Arabidopsis, *Orobanche*, and AM fungi have distinct structural requirements and thus act through slightly modified SL receptors, resulting in the variations in activity.

The CD-Rings of the SL Analogs

The CD-rings and the enol ether connecting them seem to be critical for SL function, but also a point of contrast between the SL-responding organisms. For example, whereas GR5 (missing both A- and B-rings) is able to stimulate *Orobanche* seed germination, inhibit shoot-bud outgrowth, and induce RH elongation, it is incapable of inducing hyphal branching (Johnson et al., 1976; Akiyama et al., 2010; Boyer et al., 2012). Moreover, imino analogs of GR24, in which the enol ether is lacking, have been found to be partly active on *Orobanche* seed germination (Kondo et al., 2007) and on AM hyphal branching (Akiyama et al., 2010).

In addition, evidence is accumulating for the germination activity of molecules lacking the enol–ether moiety and the ABC-rings (e.g. karrikins or saccharin derivatives, respectively; Zwanenburg and Mwakaboko, 2011; Scaffidi et al., 2012). As a matter of fact, it might be that a leaving group on the C5 of the D-ring is sufficient to initiate the SLs' stimulatory activity (Scaffidi et al., 2012). These mechanisms are consistent with the stimulatory activity of molecules lacking the enol–ether bridge linking the C- and D-rings, and both models point to the critical role played by the butenolide D-ring (Zwanenburg and Mwakaboko, 2011; Scaffidi et al., 2012). Taken together, the activities of GR5 and GR7 observed in our system derive from the existence of a D-ring and a leaving group.

The AB-Rings of the SL Analogs

AB-ring composition plays an important role in the SL bioactivity that differed between the tested organisms. For example, the presence of a hydroxyl group on the AB part of naturally occurring SLs shows different effects on the SL response in the organisms studied here: hydroxy-SL enhances *Orobanche* seed-germination stimulation (Xie et al., 2008; Kim et al., 2010), but reduces hyphal branching activity (Akiyama et al., 2010) and shoot-branching inhibition (Boyer et al., 2012).

Here, we show that substitutions on the A-ring of the analogs ST357 and ST362 may determine their activity. Both ST molecules present a thiophene ring attached to their A part; these analogs fail to induce RH elongation of *Arabidopsis*. The principal reason for this inactivity might stem from the chemical properties of the thiophene ring, which is a heterocyclic molecule that exhibits pharmacological activities. For example, thiophene-carrying compounds can act as serotonin antagonists and anticancer agents, and they are widely employed in the design of biologically active molecules (reviewed by Mishra et al. (2011)). In contrast, EGO23 and EGO10 molecules have no thiophene nucleus and display strong activity on *Arabidopsis* RHs. With respect to activity on RHs of *Arabidopsis*, it is worth

noting the sharp distinction between the ST and EGO series. Aside from the substitution on the A-ring, the main difference between them lies in the presence (ST) or absence (EGO) of a methyl on C. Furthermore, an additional compound bearing a thiophene ring on the C6 of the A-ring, but no methyl on C, was found to be inactive on RH elongation (M. Cohen, unpublished results). Hence, the presence of a thiophene substituent on the A-ring in the ST molecules might account for their lack of activity in *A. thaliana* roots.

In contrast, the activation of weed seed SL receptor seems to be indifferent to the presence of a thiophene substituent: ST357 showed high germination induction, whereas ST362 (both thiophene-presenting analogs) showed low intrinsic activity and low affinity.

Moreover, both analogs that induced germination at a low rate, ST362 and EGO23, display high van der Waals surface areas—585 Å² and 595 Å², respectively (data provided by ChemAxon software)—compared to the other active analogs tested (e.g. GR24 and ST357, with surface areas of between 370 Å² and 517 Å²). Studies of the receptor–ligand interactions at the serotonin receptor have shown that attachment of a voluminous side group to the ligand molecule affects affinity to the receptor (López-Rodríguez et al., 2002, 2003). Therefore, the bulky side groups linked to the A-ring of the molecules ST362 and EGO23 might cause their reduced affinities to the receptor of *O. aegyptiaca*. Both EGO10 and ST357 (referred to as EGO5 and ST23c, respectively) were tested on *O. aegyptiaca* by Prandi et al. (2011) and both induced germination at rates similar to those of GR24, confirming the activity of these analogs on *Orobanche* seeds.

In contrast, in *G. intraradices*, the most active molecule is the bulky, thiophene-containing ST362. Therefore, presence of a thiophene nucleus and bulkiness of the A-ring substituent do not seem to disturb binding and activation of the SL receptor in this fungus. The analog EGO10 was tested on the fungus *Gigaspora margarita* (Prandi et al., 2011) and induced an intermediate level of hyphal branching, similar to the results obtained with the fungus *G. intraradices*. Interestingly, the less potent analog tested on the fungus *G. intraradices* (ST357/ST23c) was found to highly induce hyphal branching of the fungus *G. margarita* (Prandi et al., 2011). This divergence might indicate differences within species of AM fungi in sensitivity to SLs, similar to that which has been observed between the different populations of *Striga* and *Orobanche* (Wigchert et al., 1999; Fernandez-Aparicio et al., 2009).

Aside from their structural characteristics, an additional cause for the SL analogs' variations in activity can stem from their instability. Indeed, SLs are readily degradable into inactive products by cleavage of the enol–ether bond (Mangnus and Zwanenburg, 1992). Thus, the stability of the enol–ether bond can affect SL activities as a germination stimulant (Kim et al., 2010), as a branching factor for AM fungi (Akiyama et al., 2010), and as a plant hormone (Boyer et al., 2012). Rationally, differences in stability between the nitrogen-derivative analogs could cause the observed variations in activity. However, in preliminary experiments analyzing the formation of by-products by HPLC with time, solutions of the different analogs did not seem to show decreased concentration of the active compound within 2 weeks (C. Prandi, unpublished results). Moreover, if the stability of the molecules was the reason for their decreased activity, one would expect a decrease in all examined activities. However, this study showed that the tested analogs are active in at least one of the examined systems. Therefore, the differences in activity between the molecules do not stem from their stability but from their intrinsic activity and affinity to a putative receptor.

Antagonistic Behavior of the Analog Molecules

The *Arabidopsis* mutant impaired in the F-box protein MAX2, suspected of mediating the SL response, was insensitive to all of the analogs; it is therefore likely that, in *Arabidopsis*, the same signaling pathway is used by all of the analogs. In the sense that the ST molecules are unable to activate the perception system of *Arabidopsis* roots, they have no intrinsic activity. However, the results obtained from the competitive experiments suggest that these analogs share antagonist interactions with active molecules such as GR24 on the SL receptor in roots of *Arabidopsis*. Therefore, we suggest that ST362 and ST357 are capable of binding to the receptor but cannot activate it.

In the context of the seeds of the parasitic weed *O. aegyptiaca*, ST362 also behaves like a competitive antagonist and disturbs the binding of the full agonist molecule, GR24, to its receptor. Therefore, the A-ring's composition not only affects binding of the molecule to its receptor (by reducing its affinity), but also affects activation of the SL receptor by reducing its intrinsic activity.

Despite showing reduced activity for some of the molecules, our results on the antagonistic behavior of the analog molecules suggest that they all bind the receptor through molecular mechanisms of SL binding to its receptor.

On the one hand, Zwanenburg and co-workers proposed a mechanism whereby a Michael addition reaction of a nucleophile occurs at the butenolide D-ring instead of at the enol–ether moiety as suggested previously (Zwanenburg et al., 2009). Subsequent proton exchanges lead to covalent binding of the nucleophilic part of the receptor to the D-ring and to elimination of the group at the C2′ of the butenolide D-ring. On the other hand, Flematti proposed an alternative mechanism supporting the germination activity of karrikins (Scaffidi et al., 2012). In this case, a nucleophile at the receptor site does not react in a Michael fashion with an α/β-unsaturated system, but preferably causes opening of the lactonic D-ring. This occurs through hydrolytic activity of an α/β hydrolase suspected of being part of the SL signaling pathway (Arite et al., 2009; Waters et al., 2012). However, it is suggested that, in SL molecules, the α/β hydrolase is more likely to hydrolyze the enol–ether bond than the butenolide D-ring (Hamiaux et al., 2012).

The different molecules tested herein presented various levels of activity in each of the tested organisms. Taken together, these results suggest that the SL receptor behaves in

a structure-dependent and organism-specific manner through variations in receptor sensitivity to SL. In principle, it might be possible to synthesize molecules that will enhance or inhibit the different effects of SL (perception by AM fungi, hormone activity, and germination of parasitic seeds) to serve a variety of purposes.

On the one hand, we could make use of the common structural requirements among the tested organisms. For example, a compound like the SL analog EGO10 (EGO5 in Prandi et al. (2011)) is able to activate all tested biological systems. Its activity is similar to that of GR24 in seeds of *Orobanche* and in roots of *Arabidopsis*. In addition, EGO10 is the nitrogen-derivative analog whose structure is closest to that of GR24 and its synthesis requires few steps and feasible reaction conditions (Prandi et al., 2011). Therefore, a compound like the analog EGO10 could be a judicious choice for technical use instead of GR24, for manipulation of *Arabidopsis* RH elongation, *Orobanche* seed germination, and, to a lesser extent, hyphal branching of the AM fungus *G. intraradices.*

On the other hand, the analogs' specificity for a particular organism might also be exploited. For example, the compound ST357 strongly induces weed seed germination but has no effect on the plant system. Therefore, this analog could be safely used as a germination stimulant to combat the detrimental effect of parasitic weeds without affecting the plant hormonal system. To conclude, this study provides an assessment and better understanding of the structural requirements of SL for its different functions.

METHODS

Synthesis

SL analogs ST357, ST362, EGO10, and EGO23 were prepared as previously reported (Prandi et al., 2011). GR24, GR5, and GR7 were prepared as reported by Johnson et al. (1981) and (1976), respectively.

Orobanche Germination Assay

Approximately 30–50 seeds of *O. aegyptiaca*, surface-sterilized with 70% ethanol solution, 1% (v/v) sodium hypochlorite, 0.1% benlate (v/v), and distilled water, were spread on a glass-fiber filter paper disk (9-mm diameter) and put into sterile Petri dishes (9-cm diameter) lined with Whatman filter paper wetted with 3 ml of de-mineralized water. Petri dishes were sealed with parafilm and incubated for preconditioning. After 1 week of preconditioning, the glass-fiber filter paper disks with *O. aegyptiaca* seeds were removed from the Petri dish and dried for 20 min to remove excess moisture. The disks were then transferred to another Petri dish within a filter paper ring (outer diameter of 9 cm; inner diameter of 8 cm) wetted with 1 ml water, which maintained a moist environment during the germination bioassay. Test solutions (40 µl) were added to triplicate disks; four triplicate disks were used per treatment (n = 350). Test samples were first dissolved in acetone then diluted with distilled sterilized water to a final acetone content of 1:1,000 (v/v). In each experiment, a negative control with 0.1% acetone was run. Means of replicates were subjected to one- or two-way ANOVA by multiple-range Tukey–Kramer test ($P \leq 0.05$) using the JMP statistical package. All experiments were repeated three times.

Arabidopsis Growth Conditions

Seeds of *Arabidopsis* wild-type (WT; Columbia, Col-0) were surface-sterilized and germinated on half-strength Murashige and Skoog (MS) plates supplemented with 1.5% (w/v) sucrose. Plates were incubated vertically in the dark at 4°C for 2 d to synchronize germination. Three days after germination, seedlings were gently transferred using forceps to half-strength MS plates containing various concentrations of analogs or controls. Test samples were first dissolved in acetone then diluted with distilled sterilized water and further diluted in 20 ml half-strength MS to a final acetone content of 1:1,000 (v/v). A negative control with 0.1% acetone was run in each experiment. The location of the root tip of the transferred seedling was marked on the plate. The plate was left unsealed to prevent accumulation of gases (e.g. ethylene). Plates were positioned in an upright 45° position, and incubated at 22°C with a light intensity of 50–60 µE m^{-2} s^{-1} provided by white fluorescent tubes under a 16-h light/8-h dark photoperiod for 6–12 d.

Determination of *Arabidopsis* RH Length

For examination of RH length, roots were grown on treatment and control plates as described above. Following 2 d of growth, roots were examined on the plates using a stereomicroscope. Pictures of root segments that had grown on the plates for 48 h under the examined conditions and were in the mature part of the root were taken with a Nikon DS-Fi1 camera. Measurements of RH length were performed on 15 pictures per treatment; between 15 and 20 RHs were measured per picture, using IMAGEJ (n = 225). Means of replicates were subjected to one- or two-way ANOVA by multiple-range Tukey–Kramer test ($P \leq 0.05$) using the JMP statistical package. All experiments were repeated three times.

Mycorrhizal Fungi Hyphal Branching Assay

Mycorrhizal studies were conducted with the AM fungus *G. intraradices* (LPA 8). Ten spores were individually transferred to Petri dishes containing M medium (0.4% w/v gelzan) and incubated to germination at 32°C in a 2% CO_2 atmosphere for 1 week. A Pasteur pipette was used to make two small holes on both sides of the growing primary hypha. The holes were then filled with 5 µl aqueous solution containing the different analogs. Test samples were first dissolved in acetone then diluted with sterilized water to a final acetone content of 1:1000 (v/v). Controls including 0.1% acetone and GR24 were also run. After 7 d of growth at 32°C in a 2% CO_2 atmosphere, hyphae were stained with Trypan blue and primary and secondary branches were counted (n =150). Means of 15 replicates were subjected to one- or two-way ANOVA by multiple-range Tukey–Kramer test ($P \leq 0.05$) using the JMP statistical package. The experiment was repeated three times.

Calculation of Logistic Dose–Response Curve

The dose–response curves were obtained using nonlinear regression performed by Graphpad Prism 5 software. SL analog-induced responses can be studied quantitatively by analogy to the Michaelis-Menten model of enzyme kinetics (Wigchert et al., 1999; Matusova et al., 2004). Logistic dose–response curves describe sensitivity by defining three sensitivity parameters (Weyers et al., 1987): R_{max}, EC_{50}, and p. R_{max} is the maximal response and relates to the intrinsic activity of an agonist (Ariens et al., 1983). EC_{50} is the concentration required to get half the maximal response, and p is the interaction or Hill coefficient. Both EC_{50} and p describe affinity of the compound to the receptor: $R = R_{min} + \frac{R_{max} - R_{min}}{1 + ([EC]_{50}/[EC])^p}$

where R_{min} is germination in the absence of any stimulant and [*EC*] is the applied dose.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

ACKNOWLEDGMENTS

We are grateful to Dr Kohki Akiyama from Osaka Prefecture University for supplying us with the SL analogs GR5 and GR7, and Dr Koichi Yoneyama from Utsunomiya University for supplying us with the SL analog GR24. No conflict of interest declared.

REFERENCES

Akiyama, K. (2007). Chemical identification and functional analysis of apocarotenoids involved in the development of arbuscular mycorrhizal symbiosis. Biosci. Biotechnol. Biochem. **71**, 1405–1414.

Akiyama, K., Matsuzaki, K., and Hayashi, H. (2005). Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. Nature. **435**, 824–827.

Akiyama, K., Ogasawara, S., Ito, S., and Hayashi, H. (2010). Structural requirements of strigolactones for hyphal branching in AM fungi. Plant Cell Physiol. **51**, 1104–1117.

Arite, T., Umehara, M., Ishikawa, S., Hanada, A., Maekawa, M., Yamaguchi, S., and Kyozuka, J. (2009). d14, a strigolactone-insensitive mutant of rice, shows an accelerated outgrowth of tillers. Plant Cell Physiol. **50**, 1416–1424.

Besserer, A., Bécard, G., Jauneau, A., Roux, C., and Séjalon-Delmas, N. (2008). GR24, a synthetic analog of strigolactones, stimulates the mitosis and growth of the arbuscular mycorrhizal fungus *Gigaspora rosea* by boosting its energy metabolism. Plant Physiol. **148**, 402–413.

Besserer, A., Puech-Pages, V., Kiefer, P., Gomez-Roldan, V., Jauneau, A., Roy, S., Portais, J.C., Roux, C., Becard, G., and Sejalon-Delmas, N. (2006). Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria. PLoS Biol. **4**, 1239–1247

Beveridge, C.A., Ross, J.J., and Murfet, I.C. (1996). Branching in pea (action of genes *Rms3* and *Rms4*). Plant Physiol. **110**, 859–865.

Bhattacharya, C., Bonfante, P., Deagostino, A., Kapulnik, Y., Larini, P., Occhiato, E., Prandi, C., and Venturello, P. (2009). A new class of heterocyclic strigolactone analogues with fluorescent properties: synthesis and biological activity. Org. Biomol. Chem. **7**, 3413–3420.

Boyer, F.D., Saint Germain, A., Pillot, J.P., Pouvreau, J.B., Xiao Chen, V., Ramos, S., Stevenin, A., Simier, P., Delavault, P., Beau, J.M., et al. (2012). Structure–activity relationship studies of strigolactone-related molecules for branching inhibition in garden pea: molecule design for shoot branching. Plant Physiol. **159**, 1524–1544.

Butler, L.G. (1995). Chemical communication between the parasitic weed *Striga* and its crop host: a new dimension in allelochemistry. Allelopathie. **12**, 158–166.

Cook, C.E., Whichard, L.P., Turner, B., Wall, M.E., and Egley, G.H. (1966). Germination of witchweed (*Striga lutea* Lour.): isolation and properties of a potent stimulant. Science. **154**, 1189–1190.

Cook, C.E., Whichard, L.P., Wall, M.E., Egley, G.H., and Coggon, P. (1972). Germination stimulants. II. The structure of strigol—a potent seed germination stimulant for witchweed (*Striga lutea* Lour.). J. Am. Chem. Soc. **94**, 6198–6199.

Dun, E.A., Brewer, P.B., and Beveridge, C.A. (2009). Strigolactones: discovery of the elusive shoot branching hormone. Trends Plant Sci. **14**, 364–372.

Fernandez-Aparicio, M., Flores, F., and Rubiales, D. (2009). Recognition of root exudates by seeds of broomrape (*Orobanche* and *Phelipanche*) species. Annals of Botany. **103**, 423–431.

Gomez-Roldan, V., Fermas, S., Brewer, P.B., Puech-Pagès, V., and Dun, E.A. (2008). Strigolactone inhibition of shoot branching. Nature. **455**, 189–194.

Hamiaux, C., Drummond, R.S.M., Janssen, B.J., Ledger, S.E., Cooney, J.M., Newcomb, R.D., and Snowden, K.C. (2012). DAD2 is an α/β hydrolase likely to be involved in the perception of the plant branching hormone, strigolactone. Curr. Biol. **22**, 2032–2036.

Hauck, C., Müller, S., and Schildknecht, H. (1992). A germination stimulant for parasitic flowering plants from *Sorghum bicolor*, a genuine host plant. J. Plant Physiol. **139**, 474–478.

Ishikawa, S., Maekawa, M., Arite, T., Onishi, K., Takamure, I., and Kyozuka, J. (2005). Suppression of tiller bud activity in tillering dwarf mutants of rice. Plant Cell Physiol. **46**, 79–86.

Johnson, A.W., Gowda, G., Hassanali, A., Knox, J., Monaco, S., Razavi, Z., and Rosebery G. (1981). The preparation of synthetic analogues of strigol. J. Chem. Soc. Perkin Trans. **1**, 1734–1743.

Johnson, A.W., Rosebery, G., and Parker, C. (1976). A novel approach to *Striga* and *Orobanche* control using synthetic germination stimulants. Weed Res. **16**, 223–227.

Kapulnik, Y., Delaux, P.M., Resnick, N., Mayzlish-Gati, E., Wininger, S., Bhattacharya, C., Séjalon-Delmas, N., Combier, J.P., Bécard, G., and Belausov, E. (2011). Strigolactones affect lateral root formation and RH elongation in *Arabidopsis*. Planta. **233**, 209–216.

Kim, H.I., Xie, X., Kim, H.S., Chul, C.J., Yoneyama, K., and Nomura, T. (2010). Structure–activity relationship of naturally occurring strigolactones in *Orobanche minor* seed germination stimulation. J. Pestic. Sci. **35**, 344–347.

Kohlen, W., Charnikhova, T., Liu, Q., Bours, R., Domagalska, M.A., Beguerie, S., Verstappen, F., Leyser, O., Bouwmeester, H., and Ruyter-Spira, C. (2011). Strigolactones are transported through the xylem and play a key role in shoot architectural response

to phosphate deficiency in nonarbuscular mycorrhizal host *Arabidopsis*. Plant Physiol. **155**, 974–987.

Koltai, H. (2011). Strigolactones are regulators of root development. New Phytol. **190**, 545–549.

Koltai, H., Dor, E., Hershenhorn, J., Joel, D., and Weininger, S. (2010). Strigolactones' effect on root growth and root-hair elongation may be mediated by auxin-efflux carriers. J. Plant Growth Regul. **29**, 129–136.

Kondo, Y., Tadokoro, E., Matsuura, M., Iwasaki, K., and Sugimoto, Y. (2007). Synthesis and seed germination stimulating activity of some imino analogs of strigolactones. Biosci. Biotechnol. Biochem. **71**, 2781–2786.

López-Rodríguez, M.L., Benhamú, B., Morcillo, M.J., Murcia, M., Viso, A., Alvaro, E., Campillo, M., and Pardo, L. (2003). The recognition of the voluminous substituent attached to the basic amino group of 5-HT4 receptor antagonists J. Computer-Aided Mol. Design. **17**, 515–524.

López-Rodríguez, M.L., Benhamú, B., Morcillo, M.J., Murcia, M., Viso, A., Campillo, M., and Pardo, L. (2002). 5-HT4 receptor antagonists: structure–affinity relationships and ligand– receptor interactions. Curr. Top. Med. Chem. **2**, 625–641.

Mangnus, E.M., and Zwanenburg, B. (1992). Tentative molecular mechanism for germination stimulation of *Striga* and *Orobanche* seeds by strigol and its synthetic analogs. J. Agric. Food Chem. **40**, 1066–1070.

Matusova, R., Rani, K., Verstappen, F.W., Franssen, M.C., Beale, M.H., and Bouwmeester, H.J. (2005). The strigolactone germination stimulants of the plant-parasitic *Striga* and *Orobanche* spp. are derived from the carotenoid pathway. Plant Physiol. **139**, 920–934.

Matusova, R., Van Mourik, T., and Bouwmeester, H.J. (2004). Changes in the sensitivity of parasitic weed seeds to germination stimulants. Seed Sci. Res. **14**, 335–344.

Mishra, R., Jha, K.K., Kumar, S., and Tomer, I. (2011). Synthesis, properties and biological activity of thiophene: a review. Der Pharma Chemica. **3**, 38–54.

Müller, S., Hauck, C., and Schildknecht, H. (1992). Germination stimulants produced by *Vigna unguiculata* Walp cv Saunders Upright. J. Plant Growth Regul. **11**, 77–84.

Prandi, C., Occhiato, E., Tabasso, S., Bonfante, P., Novero, M., Scarpi, D., Bova, M., and Miletto, I. (2011). New potent fluorescent analogues of strigolactones: synthesis and biological activity in parasitic weed germination and fungal branching chemistry. Eur. J. Org. Chem. **21**, 3781–3793.

Reizelman, A., Wigchert, S.C.M., del-Bianco, C., and Zwanenburg, B. (2003). Synthesis and bioactivity of labelled germination stimulants for the isolation and identification of the strigolactone receptor. Org. Biomol. Chem. **1**, 950–959

Ruyter-Spira, C., Kohlen, W., Charnikhova, T., van Zeijl, A., van Bezouwen, L., de Ruijter, N., Cardoso, C., Lopez-Raez, J.A., Matusova, R., and Bours, R. (2011). Physiological effects of the synthetic strigolactone analog GR24 on root system architecture in *Arabidopsis*: another below-ground role for strigolactones? Plant Physiol. **155**, 721–734.

Scaffidi, A., Waters, M.T., Bond, C. S., Dixon, K.W., Smith, S.M., Ghisalberti, E.L., and Flematti, G.R. (2012). Exploring the molecular mechanism of karrikins and strigolactones Bioorg. Med. Chem. Lett. **22**, 3743–3746.

Stirnberg, P., Furner, I.J., and Leyser, O. (2007). MAX2 participates in an SCF complex which acts locally at the node to suppress shoot branching. Plant J. **50**, 80–94.

Stirnberg, P., Van De Sande, K., and Leyser, O. (2002). MAX1 and MAX2 control shoot lateral branching in *Arabidopsis*. Development. **129**, 1131–1141.

Thuring, J., Keltjens, R., Nefkens, G.H.L., and Zwanenburg, B. (1997). Synthesis and biological evaluation of potential substrates for the isolation of the strigol receptor. Journal of the Chemical Society–Perkin Transactions. **1**, 759–765.

Umehara, M., Hanada, A., Yoshida, S., Akiyama, K., and Arite T. (2008). Inhibition of shoot branching by new terpenoid plant hormones. Nature. **455**, 195–200.

Waters, M.T., Nelson, D.C., Scaffidi, A., Flematti, G.R., and Sun, Y.M. (2012). Specialisation within the DWARF14 protein family confers distinct responses to karrikins and strigolactones in *Arabidopsis*. Development. **139**, 1285–1295.

Weyers, J.D.B., Paterson, N.W., and A'Brook, R. (1987). Toward a quantitative definition of plant hormone sensitivity. Plant Cell Environ. **10**, 1–10.

Wigchert, S.C.M., Kuiper, E., Boelhouwer, G.J., Nefkens, G.H.L., Verkleij, J.A.C., and Zwanenburg, B. (1999). Dose–response of seeds of the parasitic weeds *Striga* and *Orobanche* toward the synthetic germination stimulants GR 24 and Nijmegen 1. J. Agric. Food Chem. **47**, 1705–1710.

Xie, X., Yoneyama, K., and Yoneyama, K. (2010). The strigolactone story. Phytopathology. **48**, 93–117.

Xie, X., Yoneyama, K., Kusumoto, D., Yamada, Y., Yokota, T., Takeuchi, Y., and Yoneyama, K. (2008). Isolation and identification of alectrol as (+)-orobanchyl acetate, a germination stimulant for root parasitic plants. Phytochemistry. **69**, 427–431.

Zwanenburg, B., and Mwakaboko, A.S. (2011). Strigolactone analogues and mimics derived from phthalimide, saccharine, *p*-tolylmalondialdehyde, benzoic and salicylic acid as scaffolds. Bioorganic and Medicinal Chemistry. **19**, 7394–7400.

Zwanenburg, B., Mwakaboko, A.S., Reizelman, A., Anilkumar, G., and Sethumadhavan, D. (2009). Structure and function of natural and synthetic signaling molecules in parasitic weed germination. Pest Manage. Sci. **65**, 478–491.